Aquaculture Research, 1997, 28:809-814

Increasing cyst yields in *Artemia* culture ponds in Vietnam: the multi-cycle system

P. Baert[1], N.T.N. Anh[1], Vu Do Quynh[1], N.V. Hoa[1], and P. Sorgeloos[2]

[1] Shrimp and *Artemia* R&D Institute (SARDI)
Can Tho University, Can Tho, Vietnam

[2] Laboratory of Aquaculture & *Artemia* Reference Centre
University of Gent, Belgium

ABSTRACT

In this study, one-cycle and multi-cycle culture systems for *Artemia* in seasonal salt ponds are compared. In one-cycle systems, *Artemia* is inoculated only once per season, while in multi-cycle systems ponds are drained and re-stocked several times per season. In Vietnam, three-cycle systems gave significantly higher cyst yields than did the one-cycle system. However, after two cycles, systems were not significantly different. Food limitation probably caused the steady decline in cyst yields, as observed in the one-cycle ponds. In these ponds, females have smaller broods (from the second cycle onwards) and during the last cycle the number of adult females is lower than in multi-cycle ponds.

Aquaculture Nutrition 1997 3, 39–47

Dietary phosphatidylcholine requirements in larval and postlarval *Penaeus japonicus* Bate*

M.R. CAMARA[1], P. COUTTEAU & P. SORGELOOS *Laboratory of Aquaculture & Artemia Reference Center, University of Ghent, Gent, Belgium*

*This paper forms part of the Fifth Symposium of the International Working Group on Crustacean Nutrition published in *Aquaculture Nutrition* 2, 3.

Abstract

Three nutritional experiments were carried out to investigate the effect of dietary phosphatidylcholine (PC) on the growth (length, weight and metamorphosis) and survival (rearing and stress) of larval and postlarval *Penaeus japonicus* Bate. Purified soybean phosphatidylcholine (SPC, 95% purity), hen egg-PC (EPC, 94% purity), and de-oiled soybean lecithin (DSL, PC 23% purity) were used as sources of dietary PC. The results indicated that optimal metamorphosis in larval *P. japonicus* was obtained at levels of 15–30 g kg^{-1} of dietary SPC. Feeding trials with postlarval *P. japonicus* showed that low levels of dietary SPC (15 g kg^{-1}) were more beneficial than higher levels of SPC (30 g kg^{-1}) or DSL (65 g kg^{-1}), although early postlarvae (<3 mg dry weight) presented a higher requirement for PC than later stages. Shrimp fed the 150 g kg^{-1} PC-supplemented diets exhibited significantly better resistance to salinity stress than those fed a PC-deficient diet. A high level of dietary soybean lecithin (65 g kg^{-1} DSL), providing 15 g kg^{-1} of dietary PC, was no more effective for young postlarvae of *P. japonicus* than 15 g kg^{-1} of purified PC alone, which suggested that the other phospholipids (mainly phosphatidylethanolamine and phosphatidylinositol) in the soybean lecithin are not required for postlarval shrimp, at least when there is already an adequate source of PC.

KEY WORDS: lecithin, nutrition, *Penaeus japonicus*, phosphatidylcholine, phospholipid, salinity stress

Received 29 June 1995, accepted 3 June 1996

Correspondence: Dr P. Coutteau, Laboratory of Aquaculture and Artrenia Reference Center, University of Ghent, Razier 44, B–9000 Gent, Belgium.
[1]Present address: Universidade Federol do Ria Grande do Norte, Departamento de Oceanagrafia e Limnolagia, Natal, RN, Brazil

Introduction

Dietary phospholipids, and particularly phosphatidylcholine (PC), have been found to enhance growth and survival in penaeid shrimp (Kanazawa 1982; Kanazawa *et al.* 1985). Due to the high cost of purified phospholipids, most shrimp-feeding trials reported in the literature have been performed with phospholipids of unspecified composition or low purity (Kanazawa *et al.* 1979; Piedad-Pascual 1986; Teshima *et al.* 1986; Kanazawa 1993). This makes it difficult to draw conclusions with regard to the optimal levels/types of dietary phospholipids required and impossible to compare different studies. Relatively few studies have evaluated precise requirements using highly purified phospholipid sources (Chen & Jenn 1991; Chen 1993). Furthermore, very different responses to feeding phospholipids have been noticed in crustaceans in relation to the growth stage of the animals and the composition of the other dietary ingredients (Kanazawa *et al.* 1985).

The present study aimed at the evaluation of the dietary effect of highly purified soybean phosphatidylcholine (95% purity) on growth and survival of larval and early postlarval shrimp, *Penaeus japonicus* Bate, fed semi-purified diets. In addition to production parameters, the overall physiological status of the animals was assessed using a stress test based on exposure to osmotic shock. To facilitate interspecies comparisons, the phospholipid sources and experimental design used in this study were identical to those in a parallel study with postlarval *P. vannamei* (Coutteau *et al.* 1996).

Materials and methods

Origin and acclimation of animals

Penaeus japonicus nauplii (substage 4–5; total length 0.49 ± 0.01 mm) used in the larval rearing experiment (experiment 1) were kindly provided by Maresa, Mariscos de Esteros S.A., Ayamonte, Huelva, Spain. For the two other feeding trials (experiments 2 and 3), postlarval *Penaeus japonicus* (4-to-6-day-old postlarvae) were obtained from S.C.A. Mari Aude, Leucate, France.

Animals were delivered in oxygen-inflated 10-L plastic bags, filled with 5 L of cooled seawater (16–20°C; 25–27 g L^{-1}) and

placed in styrofoam boxes. Upon arrival, shrimp were gradually acclimated to the laboratory conditions. Shrimp nauplii were transferred immediately after acclimation to the larval rearing system whereas postlarvae were kept for 2 days in the acclimation tank and fed freshly hatched *Artemia* nauplii (EG/DECAR, INVE Aquaculture N.V., Baasrode, Belgium). To compensate for possible stress effects of handling animals during stocking procedures, mortalities were replaced during the first 24 h of the experiments.

Culture of larvae

The larval experiment (experiment 1) was conducted in 1-L cylindro-conical glass tubes (height 45 cm, diameter at top, 10.5 cm) similar to the Imhoff cone system used for larviculture of several penaeid shrimp species (Wilkenfeld *et al.* 1983, 1984; Biedenbach *et al.* 1989; Smith *et al.* 1992). The glass tubes were mounted in a water bath equipped with thermostatic heaters and circulation pumps. Aeration for each cone was provided by 1-mL disposable plastic pipettes reaching to the bottom of the tube. Continuous overhead illumination was provided by 40-W cool white fluorescent light tubes. Sea water and food in each glass tube were replaced every day by pouring the entire contents of the tube through a sieve which collected the larvae. Animals were kept submerged by holding the screen in a Petri dish during the whole exchange procedure. Before being replaced into the glass tubes, shrimp were observed and their developmental stage determined under a dissecting microscope. Larval stages were identified as described by Hudinaga (1942). The glass tube was then refilled with fresh sea water and the larvae washed back from the collecting screen into the tube, and the different feeds supplied. The initial stocking density in each cone was 100 shrimp L^{-1}. The experiment was run with eight replicates and terminated when 90% of the larvae in any treatment group metamorphosed to the postlarval stage (this occurred after 11 days of culture).

Culture of postlarvae

The two experiments with postlarval *P. japonicus* (initially approximately 1 mg dry body weight, 10 mm total length; experiments 2 and 3) were performed in a water recirculation system comprising a series of aquaria (50 × 20 × 25 cm, useful volume of 20 L; white bottom and grey walls) equipped with independent biological filters (12-L volume) over which the sea water was continuously recirculated by an air-water lift at a rate of 1 L min^{-1} (Fig. 1). Sand-filtered natural sea water was purified by passing through a 5-μm filter bag and Na_2EDTA (10 mg L^{-1}) was added 24 h prior to use. Salinity and temperature in each tank were kept

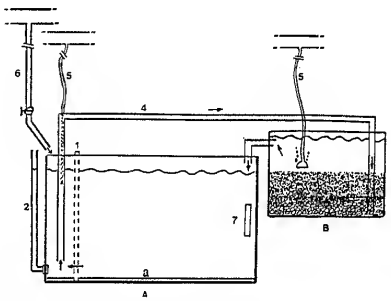

Figure 1 Diagram of one culture unit (A) and its biological filter (B) used for postlarval rearing of *Penaeus japonicus* (1: screen, 2: water outlet, 3: white bottom, 4: air–water lift, 5: air-supply, 6: inlet for filtered sea water, 7: thermostatic heater).

at 30 g L^{-1} and 27°C, respectively. Temperature was controlled with a 100-W thermostatic heater, and the water circulation provided adequate levels of dissolved oxygen. Approximately 10% of the water in each aquarium was replaced every day with fresh sea water. Whenever necessary the salinity was adjusted with de-ionized water to compensate for evaporation.

Values for salinity, temperature, total ammonia nitrogen, nitrite nitrogen and dissolved oxygen ranged from 28.1 to 35.3 g L^{-1}, 25.8 to 28.7°C, 0.04 to 0.09 mg L^{-1}, 0.02 to 0.11 mg L^{-1}, and 5.9 to 6.1 mg O_2 L^{-1}, respectively. Based on the water-quality parameters reported for penaeid shrimp (Wickins 1976), these results should not have adversely affected the growth and survival of shrimp throughout the experiments. The photoperiod was automatically set for a 12:12 h light:darkness cycle.

Experiment 2 was run with five replicate aquaria per treatment and postlarvae were reared in two successive phases of 17 and 14 days, respectively, at an initial stocking density of 10 and 4 shrimp L^{-1}, respectively. For experiment 3, four replicates were run and postlarvae were reared in two successive phases of 18 and 26 days, respectively (initial stocking density was 10 and 2.5 animals L^{-1}, respectively).

Postlarval shrimp were fed *ad libitum* three times a day (0900, 1300 and 1700 h). The daily food ration, corresponding to 100% of the total biomass at the beginning of each experiment, was adjusted according to the amount of food left over every day. Faeces and residues of uneaten feed were siphoned daily prior to the first feeding. The postlarvae were fed with particles of 300–500 μm in size for the first 8 days of each experiment and 500–800 μm size thereafter.

Diet preparation

Live food used, i.e. *Chaetoceros gracilis* and newly hatched *Artemia* nauplii, were produced following standardized procedures described in Wyban & Sweeney (1991) and Sorgeloos *et al.* (1986), respectively. For the larval experiment, two types of diets were prepared by INVE Aquaculture N.V./S.A. (Baasrode, Belgium) using proprietary technology, i.e. yeast-based diets and microparticulate diets. The commercial formulations of these diets served as food for the 'commercial diet control' treatment. To reduce the background supply of phospholipids through the yeast-based diets, an experimental yeast-based diet (Y[-PL]) was formulated with an identical formulation to the commercial diet, except that no additional phospholipids were present other than the endogenous yeast phospholipids. Three casein-based microparticulate diets (SPC 0, SPC 1.5 and SPC 3.0; Tables 1 and 2) were formulated to contain, respectively, 0 g kg^{-1}, 15 g kg^{-1} and 30 g kg^{-1} of purified soybean phosphatidylcholine (95% PC).

For the postlarval trials, microbound diets (MBD) were prepared according to a method and formulation modified from Teshima *et al.* (1982a). The dry ingredients were first finely ground (< 100 μm), weighed, and manually mixed in a glass bowl. The lipid components were then added drop by drop while the mixture was further blended to ensure homogeneity. κ-carrageenan (40 g kg^{-1}) was chosen as a binder because of its water-binding capacity and its reactivity with milk casein (Bautista 1990). Approximately 150 mL of distilled water at 80°C was added for each 100 g of this mixture to form a stiff dough, which was hot-extruded in a food processor (Kenwood Major KM230) with a modified meat mincer (2.5 cm diameter disc with holes of 2 mm). Pellets were subsequently dried at 25°C for 24 h to approximately 50 g kg^{-1} moisture content. Dried strands were crumbled and sieved to the desired particle size. The diets were vacuum packed and stored at –20°C until use. Small quantities of diet were refrigerated between feedings during experiments.

For the preliminary postlarval trial (experiment 2), two casein-based microbound diets were formulated to contain, respectively 0% and 3% purified soybean PC (Table 3). The formulation of the basal semi-purified diet was modified for experiment 3 by adding arginine and antioxidants, increasing the casein to 500 g kg^{-1} and the use of 50 g kg^{-1} ethyl esters instead of 85 g kg^{-1} methyl esters as source of n-3 highly unsaturated fatty acids (Table 1). The various experimental diets were derived from the basal diet and formulated to include different levels and sources of phosphatidylcholine, i.e. soybean PC (950 g kg^{-1} PC), hen-egg PC (940 g kg^{-1} PC), and de-oiled soybean lecithin (230 g kg^{-1} PC, 210 g kg^{-1} phosphatidylethanolamine, 190 g kg^{-1} phosphatidylinositol, 60 g kg^{-1} phosphatidic acid, 150 g kg^{-1} other phospholipids). Diets were made isolipidic by varying the level of refined

Table 1 Ingredient composition of the semi-purified diets for experiments 1 and 3

Ingredient	g kg^{-1}
Casein[1]	500
Arginine[2]	10
Sucrose[3]	50
Cellulose[4]	49.55
Wheat starch[5]	100
Vitamin mix[6]	20
Mineral mix[6]	100
Vitamin C[7]	10
Vitamin E[8]	0.2
κ-carrageenan[9]	40
Cholesterol[10]	5
Fish oil FO50[11]	50
Lipid mixture[12]	65
Butylated hydroxytoluene[13]	0.05
Butylated hydroxyanisole[13]	0.05
Ethoxyquin[14]	0.15

[1]Protevit K 4,3, Dena, Germany;
[2]Sigma Chemical Co., USA: A-5131;
[3]Sigma S-9378;
[4]Sigma C-8002;
[5]Sigma S-5127;
[6]Teshima *et al.* (1982a);
[7]Mg-L-ascorbyl-2-monophosphate, Phosphitan C, Showa Denko K.K., Japan;
[8]dl-α-tocopherol-acetate, Roche N.V., Belgium;
[9]Sigma C-1013;
[10]Sigma C-8503;
[11]Ethyl ester concentrate with 523 mg g^{-1} (n–3) HUFA, 301 mg g^{-1} EPA, 189 mg g^{-1} DHA; INVE Aquaculture N.V., Belgium;
[12]see Table 2;
[13]Federa N.V., Belgium;
[14]1,2-dihydro-6-ethoxy-2,2,4-trimethylquinolin, Sigma E-8260.

Table 2 Formulation of experimental diets containing different levels and sources of phosphatidylcholine (experiments 1 and 3)

Ingredient (g kg^{-1})	Treatments[1]				
	SPC 0.0	EPC 1.5	SPC 1.5	SPC 3.0	DSL 6.5
Basal diet[2]	935	935	935	935	935
Soybean oil[3]	65	50	50	35	–
Soybean phosphatidylcholine[4]	–	–	15	30	–
Egg phosphatidylcholine[5]	–	15	–	–	–
De-oiled soybean lecithin[6]	–	–	–	–	65

[1]SPC, soybean phosphatidylcholine; EPC, hen-egg phosphatidylcholine; DSL, de-oiled soybean lecithin.
[2]See Table 1.
[3]Vandemoortele N.V., Belgium.
[4]Epikuron 200, Lucas Meyer GmbH & Co., Germany.
[5]Ovothin 200, Lucas Meyer GmbH & Co., Germany.
[6]EMULPUR N, Lucas Meyer GmbH & Co., Germany.

soybean oil depending on the amount of phospholipid source included (Table 2). The water stability of the diets varied from approximately 80% after 10 min to 45% after 180 min of immersion in de-ionized water, and was not affected by changing the level and type of phospholipid (Camara 1994).

Table 3 Formulation of experimental microbound diets containing 0 g kg^{-1} and 30 g kg^{-1} PC (experiment 2)

Ingredient (g kg^{-1})	PC 3.0	PC 0
Casein[1]	450	450
Sucrose[1]	100	100
Wheat starch[1]	100	100
Fish oil FO[2]	85	85
Soybean oil[3]	–	30
Soybean phosphatidylcholine[4]	30	–
Cholesterol[5]	5	5
Mineral mix[6]	100	100
Vitamin mix[6]	20	20
Vitamin C[7]	10	10
Cellulose[1]	60	60
κ-carrageenan[1]	40	40

[1]Sigma Chemical Co., USA;
[2]Methyl ester concentrate with 465 mg g^{-1} (n–3) HUFA, 195 mg g^{-1} EPA, 242 mg g^{-1} DHA, INVE Aquaculture N.V., Belgium;
[3]Vandemoortele N.V., Belgium;
[4]Epikuron 200, Lucas Meyer GmbH & Co., Germany;
[5]Duphar B.V., The Netherlands;
[6]Teshima *et al.* (1982a);
[7]Mg-L-ascorbyl-2-monophosphate, Phosphitan C, Showa Denko K.K., Japan.

Dietary treatments

Five dietary treatments were run in the larval experiment: a live food control (LFC; fed *Chaetoceros gracilis* and *Artemia* nauplii), a commercial diet control (CDC) and three experimental treatments containing different dietary levels of soybean PC from zoea II stage onwards (experiment 1; Table 4). To minimize the background supply of phospholipids in the experimental treatments through the algae and yeast-based diets, feeding *C. gracilis* was limited to the zoea I stage and the yeast-based diet consisted of Y[-PL].

A preliminary postlarval trial (experiment 2) evaluated the effect of the supplementation of 30 g kg^{-1} PC in a casein-based microbound diet (Table 3). In the first rearing phase of experiment 3, the five dietary treatments consisted of a phospholipid-free diet (SPC 0), diets containing 15 g kg^{-1} and 30 g kg^{-1} of soybean PC (respectively, SPC 1.5 and SPC 3.0), 15 g kg^{-1} of hen-egg PC (EPC 1.5), and a diet containing 65 g kg^{-1} of de-oiled soybean lecithin (DSL 6.5). The latter diet contained an equivalent amount of 15 g kg^{-1} PC, besides the various other phospholipids present in lecithin. For the second rearing phase, treatments SPC 0 and EPC 1.5 were omitted. Animals from treatment SPC 1.5 were split into two groups, with one remaining on the same diet (SPC 1.5/SPC 1.5), whereas the other group was switched to the phospholipid-deficient diet (SPC 1.5/SPC 0); animals from treatment SPC 3.0 were divided into a group receiving the same concentration of soybean-PC (SPC 3.0/SPC 3.0) and a group receiving 50% less dietary soybean PC (SPC 3.0/SPC 1.5); animals previously fed on de-oiled soybean lecithin continued to receive the same diet (DSL 6.5/DSL 6.5).

Table 4 Feeding scheme for larval *P. japonicus* (daily amounts per mL of culture; live and artificial diets fed, respectively, once and twice daily)

	Zoea I	Zoea II	Zoea III	Mysis I	Mysis II	Mysis III
Live food control (LFC)						
C. gracilis (cells)	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5
Artemia (ind)	–	–	1 × 1.5	1 × 3	1 × 4	1 × 5.5
Commercial diet control (CDC) and experimental treatments (ET)[1]						
C. gracilis (cells)	1×10^5	–	–	–	–	–
Yeast-based diet (μg)[2]	2 × 0.5	2 × 1	2 × 2	2 × 2	2 × 1	2 × 0.5
Microparticulate diet (μg)[3]	–	2 × 8	2 × 8	2 × 8	2 × 8	2 × 8

[1]See Table 2 for the formulation of the experimental diets SPC 0, SPC 1.5 and SPC 3.0.

[2]*Treatment*	*Diet*
CDC	Commercial diet
ET 0	Y[-PL]
ET 1.5	Y[-PL]
ET 3.0	Y[-PL]

[3]*Treatment*	*Diet*
CDC	Commercial diet
ET 0	SPC 0
ET 1.5	SPC 1.5
ET 3.0	SPC 3.0

Parameters measured and statistical analysis

For the larval experiment, growth, rearing survival, and metamorphosis success were used as evaluation criteria. Body length was measured, only for animals that reached the postlarval stage, from the tip of the rostrum to the tip of the telson under a dissection microscope equipped with a drawing mirror. The projected lengths were measured with the aid of a digitizer (Graphtec, mod. KD 4300) connected to a computer. Average dry weight regardless of substage was determined by transferring all surviving shrimp per replicate to aluminum cups and oven-drying at 60°C for 24 h. The metamorphosis success was expressed as the percentage of surviving animals reaching the postlarval stage.

For the postlarval experiments, average body length and dry weight were determined as described previously at the termination of each feeding trial for 10 animals taken randomly per aquarium. Shrimp growth was expressed as an absolute increase of mean individual dry weight and as a relative increase in weight. Comparisons of growth data were made by calculating the specific growth rate (SGR, % day^{-1}) according to the equation $SGR = [(\ln W_f - \ln W_i) / t] \times 100$, where W_f and W_i are the final and initial weights, respectively, and t is the length of the culture period in days. Rearing survival was determined by counting all remaining animals at the termination of the experiment. Stress tests were performed to evaluate possible differences in the physiological condition and nutritional status of postlarval shrimp using a method modified from Tackaert *et al.* (1989). Ten animals were taken from each replicate, exposed to de-ionized water for 1 h, and put back to normal rearing conditions (30 g L^{-1} salinity and 27°C temperature). The stress survival was then assessed by counting the number of postlarvae showing active movement of the pleopods.

Data for survival (rearing survival and stress survival), growth (weight and length), and metamorphosis success were subjected to one-way analysis of variance (ANOVA) and where appropriate, Duncan's multiple range test. For experiment 2, dietary treatments were compared using a Student's *t*-test (Sokal & Rohlf 1981).

Results

The results for rearing survival, growth and metamorphosis success for the larval trial are given in Table 5. The feeding trial was terminated at day 11, when 93% of the larvae in the live diet reached metamorphosis to the postlarval stage. The live diet control treatment produced significantly better mean individual dry weight, length and rearing survival than all other treatments at the termination of the experiment. The performance of shrimp fed the commercial diet (CDC) was relatively poor in the small-scale culture system. Metamorphosis success obtained with the diet supplemented with 15 g kg^{-1} of soybean PC (ET 1.5) was not significantly different from that in the live feed control and significantly better than in the PC-deficient treatment. Conversely, shrimp fed 15 g kg^{-1} soybean PC yielded the poorest growth (dry weight and length) results within all artificial dietary treatments.

Growth, rearing survival and stress resistance of postlarval *P. japonicus* for experiments 2 and 3 are presented in Tables 6 and 7, respectively. At the end of the first culture phase of experiment 2, the mean individual weight gains of the treatment group fed the PC-supplemented diet were significantly greater than those of the group fed the PC-deficient control diet (Table 6). Although not significant, a beneficial effect of phosphatidylcholine was also noted on stress resistance, i.e. shrimp fed the PC-supplemented diet exhibited a better post-stress survival (90%) than the ones receiving the control diet (76.7%). By the end of the second phase, shrimp in the PC-deficient control treatment (PC 0) showed significantly better growth and stress survival than in the group receiving 30 g kg^{-1} PC (Table 6). There were no significant differences in rearing survival between the two treatments.

In experiment 3, the best rearing survival in the first phase was obtained in the diet containing 1.5% purified hen-egg PC (Table 7). Survival in this group was significantly better than in groups receiving different levels of purified soybean PC (SPC 1.5 and SPC 3.0) and the phospholipid-free diet (SPC 0), but not significantly different from the group receiving 65 g kg^{-1} of de-

Table 5 Mean individual dry weight, body length, rearing survival, and metamorphosis success of *P. japanicus* larvae after 1 day of culture (experiment 1). Data represent mean and standard deviation of eight replicates. Values with different superscripts in the same column are significantly different (ANOVA, Duncan, $P < 0.05$)

Treatments[1]	Dry weight (mg)	Body length (mm)	Rearing survival (%)	Metamorphosis success (% postlarvae)
LFC	$115^{a} \pm 10.0$	$4.98^{a} \pm 0.54$	$41.3^{a} \pm 11.0$	$0.93^{a} \pm 0.07$
CDC	$83^{b} \pm 7.6$	$3.83^{b} \pm 0.47$	$25.0^{b} \pm 9.2$	$0.41^{c} \pm 0.18$
ET 0	$80^{b} \pm 9.3$	$3.61^{b} \pm 0.48$	$26.0^{b} \pm 9.6$	$0.48^{c} \pm 0.20$
ET 1.5	$78^{b} \pm 8.0$	$3.17^{c} \pm 0.55$	$27.4^{b} \pm 10.7$	$0.76^{ab} \pm 0.17$
ET 3.0	$87^{b} \pm 9.1$	$3.74^{b} \pm 0.47$	$23.5^{b} \pm 6.7$	$0.62^{bc} \pm 0.17$

[1]See Table 4 for explanation.

Days of culture[1]	Treatment[2]	Weight gain (mg dry weight)	Rearing survival (%)	Stress survival (%)
17	PC 3.0	$2.97^{a} \pm 0.22$	$48.8^{a} \pm 8.2$	$90.0^{a} \pm 10.0$
	PC 0	$1.79^{b} \pm 0.13$	$54.0^{a} \pm 12.6$	$76.7^{a} \pm 5.8$
31	PC 3.0	$5.28^{b} \pm 0.75$	$66.2^{a} \pm 8.6$	$43.3^{b} \pm 15.3$
	PC 0	$6.60^{a} \pm 0.99$	$65.9^{a} \pm 17.4$	$86.7^{a} \pm 5.8$

[1]I.e. there were two successive phases, of 17 and 14 days respectively. See text.
[2]See text and Table 3 for explanation; initial postlarvae 1.3 mg dry body weight.

Table 6 Mean individual weight gain, rearing survival and stress survival of postlarval *P. japonicus* fed either a 3% PC-supplemented (PC 3.0) or a PC-deficient (PC 0) diet, after a total of 17 and 31 days of culture[1] (experiment 2). Data represent mean and standard deviation of five replicates. Within culture periods, values with different superscripts in the same column are significantly different (*t*-test; $P < 0.05$)

Phase	Treatments[1]	Weight gain (mg dry weight)	Rearing survival (%)	Stress survival (%)
I (18 days)	SPC 0	$2.37^{a} \pm 0.27$	$58.7^{b} \pm 5.2$	$16.6^{c} \pm 11.5$
	SPC 1.5	$2.89^{a} \pm 1.16$	$64.8^{b} \pm 5.8$	$70.0^{ab} \pm 10.0$
	SPC 3.0	$2.38^{a} \pm 0.15$	$61.5^{b} \pm 3.9$	$60.0^{b} \pm 10.0$
	EPC 1.5	$2.40^{a} \pm 0.17$	$76.8^{a} \pm 4.1$	$90.0^{a} \pm 10.0$
	DSL 6.5	$2.84^{a} \pm 0.61$	$73.0^{a} \pm 6.1$	$56.6^{b} \pm 5.8$
II (26 days)	SPC 1.5/SPC 0	$10.68^{ab} \pm 1.45$	$67.0^{a} \pm 6.4$	$90.0^{abc} \pm 8.2$
	SPC 1.5/SPC 1.5	$10.98^{ab} \pm 1.58$	$65.5^{a} \pm 12.9$	$95.0^{ab} \pm 5.8$
	SPC 3.0/SPC 1.5	$13.52^{a} \pm 1.03$	$76.5^{a} \pm 6.1$	$97.5^{a} \pm 5.0$
	SPC 3.0/SPC 3.0	$9.41^{b} \pm 2.30$	$64.0^{a} \pm 5.8$	$80.0^{c} \pm 0.0$
	DSL 6.5/DSL 6.5	$10.56^{b} \pm 2.02$	$72.0^{a} \pm 21.4$	$85.0^{bc} \pm 5.8$

[1]See text and Table 2 for explanation; initial postlarvae 1.0 mg dry body weight.

Table 7 Mean individual weight gain, rearing survival and stress survival of postlarval *P. japonicus* fed different dietary levels and sources of PC during two subsequent culture phases totalling 44 days (experiment 3). Data represent mean and standard deviation of four replicates. Within phases, values with different superscripts in the same column are significantly different (ANOVA, Duncan; $P < 0.05$)

oiled soybean lecithin (DSL 6.5). The hen-egg PC-fed animals also exhibited a significantly better stress resistance than animals fed other diets, except for the group receiving the 15 g kg^{-1} level of soybean PC. The phospholipid-free (SPC 0) diet gave a significantly lower stress resistance than the other treatments. During the second phase, best growth was obtained in the treatment group where the dietary level of soybean PC was lowered from 30 g kg^{-1} to 15 g kg^{-1} after the first phase (SPC 3.0/SPC 1.5; Table 7). Growth in this group was significantly better than in groups that continued to receive high levels of soybean PC (SPC 3.0/SPC 3.0) and de-oiled soybean lecithin (DSL 6.5/DSL 6.5), but not significantly different from groups that either continued to receive the lower 15 g kg^{-1} level of soybean PC (SPC 1.5/SPC 1.5) or were switched from 15 g kg^{-1} of soybean PC to the phospholipid-free diet (SPC 1.5/SPC 0). A similar trend was observed for stress survival, i.e. postlarvae in treatment SPC 3.0/SPC 1.5 exhibited a significantly better stress resistance than animals in the treatments SPC 3.0/SPC 3.0 and DSL 6.5/DSL 6.5.

Discussion

The results for growth and metamorphosis success achieved in the larval experiment for the live diet control treatment stand comparison with those reported for several other penaeid species cultured in similar experimental conditions (*P. aztecus*: Wilkenfeld *et al.* 1984; *P. vannamei* Boone: Biedenbach *et al.* 1989; *P. marginatus*, *P. monodon*, *P. schmitti*, *P. setiferus*, and *P. stylirostris*: Smith *et al.* 1992) and confirm the suitability of the small-scale culture system for the larval rearing of *P. japonicus*. The superior results obtained for the live food over existing artificial diets attest to the need for improvement before totally replacing live feed with artificial larval diets in commercial shrimp hatcheries.

D'Abramo *et al.* (1981) demonstrated that refined soybean PC was more effective in reducing mortality in the lobster *Homarus americanus* Milne Edwards than egg PC or soybean phosphatidylinositol, or non-phospholipids such as the hydrolysis products of soybean PC. Teshima *et al.* (1982b) studied the requirements of *Penaeus japonicus* larvae for phospholipids and cholesterol and observed that the inclusion of 30 g kg^{-1} soybean phosphatidylcholine (of unspecified purity) sufficed in enhancing survival and growth of the larvae from the nauplius to the postlarval stage. They suggested that the optimum levels of cholesterol and soybean phospholipids for *P. japonicus* larvae should be 10 g kg^{-1} and 30 g kg^{-1}, respectively, when pollack liver oil was used as a basal lipid source. Kanazawa *et al.* (1985) found

that the optimum level of soybean PC for growth and survival of *P. japonicus* larvae fed a semi-purified diet containing casein depended on the type of coexistent dietary lipid. The best growth and survival were attained on the diets containing 60 g kg^{-1} soybean PC (unspecified purity) when 18:1n–9 and 10 g kg^{-1} highly unsaturated fatty acids were used as basal lipids, but the inclusion of 35 g kg^{-1} soybean PC was enough to attain optimum growth and survival when pollack liver oil was used as the lipid source. Kanazawa *et al.* (1985) furthermore observed that *Penaeus japonicus* larvae reared on diets containing 70 g kg^{-1} of pollack liver oil and 10 g kg^{-1} of different sources of phospholipids presented the best growth and survival when fed 1.0% of either soybean phosphatidylcholine, bonito egg phosphatidylcholine or soybean phosphatidylinositol. However, no beneficial effect was found with 1.0% levels of dipalmitoyl-phosphatidylcholine, phosphatidylethanolamine (from bovine brain and bonito egg), phosphatidylserine and sphingomyelin (from bovine brain), cytidine-5'diphosphate choline, or taurocholic acid. They concluded that the effectiveness of the phospholipid source seemed to be dependent on the nature of the fatty acids in the a and b positions of the phospholipid molecule, and estimated the requirement for phospholipid for larval *Penaeus japonicus* to be 5–10 g kg^{-1} of the diet.

The present study confirmed the essentiality of PC for optimal metamorphosis success in larval *P. japonicus* using purified soybean phosphatidylcholine (95% purity). Although rates of metamorphosis through the different larval stages were not systematically estimated so as to minimize handling stress, the soybean-PC dietary treatment appeared also to show a better synchronization in metamorphosis throughout the experiment. This may have explained the relatively poor growth observed in the treatment fed a diet containing 15 g kg^{-1} of soybean PC. Final average weight was determined on animals of the same age but at different stages of development, whereas length measurements were made on postlarval animals only. In this way, a small fraction of older postlarvae may have yielded a higher average dry weight and length than the large fraction of synchronized younger postlarvae. As a result, growth measured as final dry weight and length may not be the best evaluation criterion for larval feeding experiments. Instead, success of metamorphosis is suggested as a better indicator of the nutritional value of a larval diet.

The postlarval trials showed that the present formulation of casein-based microbound diets could successfully be used to evaluate the dietary effect of phosphatidylcholine in penaeid shrimp. Using similar casein-based microbound diets, Kanazawa *et al.* (1977) obtained specific growth rates ranging from 6 g kg^{-1} day^{-1} to 17 g kg^{-1} day^{-1} in postlarval *P. japonicus*. Abelin *et al.* (1989) used similar experimental conditions to those in the present study and found specific growth rates ranging from 0.8% day^{-1} to 3.3% day^{-1} for postlarval *P. vannamei* fed *Artemia*-based microbound diets. More recently, Chen (1993) fed casein-based microbound diets to *P. monodon* and obtained specific growth rates ranging from 0.9% day^{-1} to 3.1% day^{-1}. Although the lack of standardization in experimental conditions (e.g. initial stage, different diet formulations, stocking rates) precludes the comparison of results of most nutrition studies with crustaceans, the specific growth rates (ranging from 6.6% day^{-1} to 7.9% day^{-1}) found in experiment 2 attest to the suitability of the formulated casein-based microbound diets for nutritional studies with penaeid shrimp.

Surprisingly, the improved growth of postlarval *P. japonicus* due to PC supplementation could only be demonstrated during the first phase of experiment 2, whereas the phospholipid-free diet performed better during the second phase. The reasons for the drastic change in the nutritional response of postlarval *P. japonicus* fed the PC-supplemented and PC-deficient diets over such a short period are unclear. While it is most likely that the younger stages of crustaceans require relatively higher levels of dietary phospholipids than the later stages (Teshima *et al.* 1982b), there are no reports indicating a negative effect associated with supplemental phospholipid for any growth stage or species of crustacean. On the contrary, also in the juvenile stages of crustaceans, a beneficial effect of dietary phospholipids has been observed (D'Abramo *et al.* 1981; Piedad-Pascual 1986; Teshima *et al.* 1986). Several investigations have suggested that the phospholipid requirements in crustaceans may vary according to the diet composition. Kanazawa *et al.* (1985) found a lower phospholipid requirement in *P. japonicus* larvae when the dietary lipid source contained high levels of (n–3) highly unsaturated fatty acids (HUFA). The dietary level of 40 g kg^{-1} (n–3) HUFA (85 g kg^{-1} of methylesters, containing 465 mg g^{-1} of (n–3) HUFA) might have lessened the dietary requirements of postlarval *P. japonicus* for phosphatidylcholine, and may explain the significantly poorer performance of the PC-supplemented diet relative to the PC-deficient diet observed during the second phase of the experiment.

The results of experiment 3 confirmed a beneficial effect of PC, either alone or in combination with other phospholipids, on stress resistance of early postlarval stages of *P. japonicus*. However, in contrast to the results obtained in experiment 2, growth in the phospholipid-supplemented treatments during the first phase of this experiment was not significantly different from that in the phospholipid-deficient diet. Although the basal diets used in both experiments were similar in composition, the inclusion of 50 g kg^{-1} of ethyl esters as the basal lipid source in the diets used in experiment 3 as compared with 85 g kg^{-1} of methyl esters for the diets of experiment 2 provided less amounts of

highly unsaturated fatty acids (26 mg g^{-1} versus 39 mg g^{-1}, respectively) as well as different DHA/EPA ratios (0.62 versus 1.24, respectively). These observations indicate that phosphatidylcholine requirements in *P. japanicus* (at least during its early postlarval stages) may vary according to the highly unsaturated fatty acid composition of the diet as suggested by Kanazawa *et al.* (1985) for larval *P. japanicus*. This assumption is further supported by the fact that shrimp fed hen-egg PC (containing 22 g kg^{-1} n–3 HUFA) in the first phase of the current experiment presented a significantly better survival rate than animals supplied with the HUFA-deficient soy PC.

The results obtained in experiment 3 also show a decreasing requirement for dietary PC with increasing age in postlarval *P. japanicus*. Low levels of PC (15 g kg^{-1}) appear to be more beneficial than higher levels (30 g kg^{-1}) of PC or de-oiled soybean lecithin (65 g kg^{-1}), with early (<3 mg dry weight) postlarvae presenting a higher requirement for PC than later stages. The trend shown in this feeding trial is consistent with the PC requirement levels reported for *P. japanicus* in other studies, i.e. values ranging from 10 g kg^{-1} of PC isolated from *Tapes phillipinarum* oil (corresponding to 88 g kg^{-1} of pure PC in the diet) for juveniles (200 mg dry weight; Kanazawa *et al.* 1979) to 30 g kg^{-1} of soybean PC (unspecified purity) for larvae (Teshima *et al.* 1982b). Likewise, the requirement of 15 g kg^{-1} PC found for early postlarval *P. japonicus* in this study is higher than those reported for juvenile stages of other penaeid species, further confirming that PC requirements may decrease in the course of development. Chen & Jenn (1991) and Chen (1993) used purified phosphatidylcholine (80% purity) as the source of phospholipids and indicated an optimum requirement of 12.5 g kg^{-1} (corresponding to 10 g kg^{-1} of pure PC) for juvenile *P. penicillatus* (200 mg dry weight) and juvenile *P. monodan* (90 mg dry weight), respectively. Kanazawa (1993) obtained the highest growth in juvenile (140 mg dry weight) *P. chinensis* with diets containing 20 g kg^{-1} soybean lecithin, corresponding to 8.4 g kg^{-1} of pure PC in the diet.

Finally, the results in experiment 3 showed that a high level (65 g kg^{-1}) of dietary soybean lecithin providing 15 g kg^{-1} of dietary PC was not more effective for young postlarvae of *P. japonicus* than purified PC alone at a 15 g kg^{-1} level. Similarly, Coutteau *et al.* (1996) found that postlarval *P. vannamei* receiving 15 g kg^{-1} of dietary phosphatidylcholine, provided either as 15 g kg^{-1} chicken-egg PC, 15 g kg^{-1} soybean PC or 65 kg^{-1} de-oiled soybean lecithin, did not show differences in growth, but had a significantly greater weight gain than that of shrimp fed 15 g kg^{-1} of de-oiled soybean lecithin (equivalent to 0.34% PC). These observations suggest that the other types of phospholipids (mainly phosphatidylethanolamine and phosphatidylinositol) contained in the soybean lecithin are not required, at least when there is already an adequate source of PC. Furthermore, a mixture of phospholipids as found in soybean lecithin does not have the same biological value as pure PC when used at the same level.

Acknowledgements

This work was supported by the Brazilian Council for Science and Technology (CNPq), the Belgian National Fund for Scientific Research NFWO (FKFO project) and the Belgian Ministry of Science Policy (GOA project). Peter Coutteau is Senior Research Assistant with the NFWO. Special thanks are due to Lucas Meyer GmbH for providing the purified phospholipid sources and to INVE Aquaculture N.V. for preparing the larval diets.

References

Abelin, P., Tackaert, W. & Sorgeloos, P. (1989) Growth response of penaeid postlarvae to dry diets containing *Artemia* biomass meal. In: *Aquaculture Europe '89* (Billard, R. & De Pauw, N. eds), pp. 3–4. European Aquaculture Society, Ostend. Special Publication no. 10,

Bautista, M.N. (1990) Use of kappa-carrageenan microbound diet (C-MB D) as feed for prawn larvae. *SEAFDEC Asian Aquaculture*, **12**, 9–11.

Biedenbach, J.M., Smith, L.L., Thomsen, T.K. & Lawrence, A.L. (1989) Use of the nematode *Panagrellus redivivus* as an *Artemia* replacement in a larval penaeid diet. *J. World Aquacult. Soc.*, **20**, 61–73.

Camara, M.R. (1994) *Dietary phosphatidylcholine requirements of* Penaeus japonicus *Bate and* Penaeus vannamei *Boone (Crustacea, Decapoda, Penaeidae)*. PhD thesis, University of Ghent, Belgium. 173 pp.

Chen, H.Y. (1993) Recent advances in nutrition of *Penaeus monodon*. *J. World Aquacult. Soc.*, **24**, 231–240.

Chen, H.Y. & Jenn, J.S. (1991) Combined effects of dietary cholesterol and phosphatidylcholine on the growth and body lipid composition of marine shrimp, *Penaeus penicillatus*. *Aquaculture*, **96**, 167–178.

Coutteau, P., Camara, M.R. & Sorgeloos, P. (1996) The effect of different levels and sources of dietary phosphatidylcholine on the growth, survival, stress resistance, and fatty acid composition of postlarval *Penaeus vannamei*. *Aquaculture*, (in press).

D'Abramo, R.L., Bordner, C.F., Conklin, D.E. & Baum, N.A. (1981) Essentiality of dietary phosphatidylcholine for survival of juvenile lobsters *Homarus*. *J. Nutr.*, **111**, 425–431.

Hudinaga, M. (1942) Reproduction, development and rearing of *Penaeus japonicus* Bate. *Jap. J. Zoal.*, **10**, 305–393.

Kanazawa, A. (1982) Penaeid nutrition. In: *Proceedings of the Second International Canference on Aquaculture Nutritian: Biachemical and Physialagical Appraaches ta Shellfish Nutrition* (Pruder, G.D., Langdon, C. & Conklin, D. eds), pp. 87–105. Rehoboth Beach, Delaware, USA. 1981. World Mariculture Society, Baian Rouge, Louisiana. Special Publication no. 2.

Kanazawa, A. (1993) Essential phospholipids of fish and crustaceans. In: *Fish Nutrition in Practice* (Kaushik, S.J. & Luquet, P. eds), Les Colloques, no. 61, pp. 519–530. INRA, Paris.

Kanazawa, A., Tokiwa, S., Kayama, M. & Hirata, H. (1977) Essential fatty acids in the diet of prawn. I. Effect of linoleic and linolenic acids on growth. *Nippon Suisan Gakkaishi*, **43**, 1111–1114.

Kanazawa, A.S., Teshima, S., Tokiwa, S., Endo, M. & Abdel Rahman, S. (1979) Effects of short-necked clam phospholipids on the growth of prawn. *Nippan Suisan Gakkaishi*, **45**, 961–965.

Kanazawa, A., Teshima, S. & Sakamoto, M. (1985) Effects of dietary lipids, fatty acids and phospholipids on growth and survival of prawn (*Penaeus japonicus*) larvae. *Aquaculture*, **50**, 39–49.

Piedad-Pascual, F.P. (1986) Effect of supplemental lecithin and lipid sources on the growth and survival of *Penaeus monodon* juveniles. In: *Proceedings 1st Asian Fish Forum* (Maclean, J.L., Dizon, L.B. & Hosillos L.V. eds), pp. 615–618. Asian Fisheries Society, Manila.

Smith, L.L., Samocha, T.M., Biedenbach, J.M. & Lawrence, A.L. (1992) Use of one-litre Imhoff cones in larviculture production. In: *Marine Shrimp Culture: Principles and Practices* (Fast, A.W. & Lester, L.J. eds), pp. 287–300. Elsevier, Amsterdam.

Sokal, R.R. & Rohlf, F.J. (1981) *Biometry. The Principles and Practice of Statistics in Biological Research*. W.H. Freeman and Company, San Francisco. 859 pp.

Sorgeloos, P., Lavens, P., Léger, P., Tackaert, W. & Versichele, D. (1986) *Manual for the Culture and Use of the Brine Shrimp* Artemia *in Aquaculture*. Artemia Reference Center, State University of Ghent, Belgium. 319 pp.

Tackaert, W., Abelin, P., Léger, P. & Sorgeloos, P. (1989) Stress resistance as a criterium to evaluate quality of postlarval shrimp reared under different feeding procedures. In: *Proceedings of the 3rd Brazilian Symposium on Shrimp Culture, Joao Pessoa, Brazil* pp. 393–403. MCR-Aquacultura Ltda, Joao Pessoa, Brazil.

Teshima, S., Kanazawa, A. & Sakamoto, M. (1982a) Microparticulate diets for the larvae of aquatic animals. *Min. Rev. Data File Fish. Res. Kagoshima Univ.*, **2**, 67–86.

Teshima, S., Kanazawa, A., Sasada, H. & Kawasaki, M. (1982b) Requirements of the larval prawn, *Penaeus japonicus*, for cholesterol and soybean phospholipids. *Mem. Fac. Fish. Kagoshima Univ.*, **31**, 193–199.

Teshima, S., Kanazawa, A. & Kakuta, Y. (1986) Effects of dietary phospholipids on growth and body composition of the juvenile prawn. *Nippon Suisan Gakkaishi*, **52**, 155–158.

Wickins, J.F. (1976) The tolerance of warm-water prawns to recirculated water. *Aquaculture*, **9**, 19–37.

Wilkenfeld, J.W., Lawrence, A.L. & Kuban, F.D. (1983) Rearing penaeid shrimp in a small-scale system for experimental purposes. In: *Proceedings of the First International Conference on Warm Water Aquaculture – Crustacea* (Rogers, G.L., Day, R. & Lim, A. eds), pp. 72–81. Brigham Young University Hawaii, Hawaii, USA.

Wilkenfeld, J.W., Lawrence, A.L. & Kuban, F.D. (1984) Survival, metamorphosis and growth of penaeid shrimp larvae reared on a variety of algal and animal foods. *J. World Mariculr. Soc.*, **15**, 31–49.

Wyban, J.A. & Sweeney, J.N. (1991) *Intensive Shrimp Production Technology. The Oceanic Institute Manual*. The Oceanic Institute, Makapuu Point, Hawaii. 157 pp.